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# Imbalanced excitatory to inhibitory synaptic input precedes motor neuron degeneration in an animal model of amyotrophic lateral sclerosis

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**Loss of motor neurons is the key neuropathological feature of amyotrophic lateral sclerosis (ALS). Although these neurons are targeted by many synapses, little is known about the importance of the pre-synaptic excitatory and inhibitory input for motor neuron degeneration. The present study aimed in determining the composition and abundance of neurochemically defined input on lumbar spinal cord motor neurons in the SOD1 mouse model of ALS by employing immunoreactivity (IR) for vesicular neurotransmitter transporter proteins. The first signs of motor neuron degeneration, visualized by vesicular acetylcholine transporter IR, were already evident in the pre-symptomatic phase at day 80 of life. With the beginning of the symptomatic phase at around day 110 of life, surviving motor neurons showed reductions in the abundances of vesicular glutamate transporter 1 and 2 appositions. This loss of excitatory input was paralleled by an essentially unchanged inhibitory input, visualized by vesicular inhibitory amino acid transporter IR. In addition, loss of excitatory and inhibitory fibers and terminals was also evident in non-motor neuron areas of the spinal cord. These data are indicative of an imbalanced synaptic input on spinal cord motor neurons with over-inhibition rather than over-excitation being a neuropathological feature during disease progression that may contribute to motor neuron death.**


*Keywords:* Vesicular transporter; Superoxide dismutase; Motor neuron; Neurodegeneration; Synaptic

## Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterized by a progressive loss of motor neurons in cortex, brain stem, and spinal cord (Cleveland and Rothstein, 2001). The degeneration of motor neurons leads to skeletal muscle weakness, paralysis, and finally death, with a mean survival of five years after disease onset. About 5–10% of ALS cases are inherited and among those about 20% are associated with missense mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) (Rosen et al., 1993). Several mechanisms have been implicated in the pathogenesis of ALS, including glutamatergic excitotoxicity, oxidative stress, mitochondrial degeneration, and defective neurofilament transport (Bruijn et al., 2004). Still, it is unclear which pathomechanism is the cause, and which is a secondary event that ultimately leads to neuronal death.

Little attention has focused on the role of the pre-synaptic components of motor neurons in the pathogenesis of ALS. Many excitatory and inhibitory synapses contact motor neurons, and a tight regulation of these inputs is the basis for normal function. Mis-regulation in either direction will lead to over-excitation or over-inhibition, both interfering with fine-tuned synaptic communication. A number of studies have addressed this issue by assessing changes in the abundance of the general synaptic vesicle marker, synaptophysin, in pre-synaptic terminals and in soma of spinal cord motor neurons from post mortem tissue of patients with sporadic ALS. Synaptophysin immunostaining was found largely reduced in the anterior horn neuropil compared to controls, but it was well preserved around the soma and the proximal dendrites of histological normal neurons (Cruz-Sanchez et al., 1996; Ikemoto et al., 1994, 2002; Matsumoto et al., 1994; Sasaki and Maruyama, 1994; Schiffer et al., 1995), even late in the disease (Ince et al., 1995). These studies suggested that alterations in synaptophysin expression occurred secondary to the lower motor neuron degeneration, but they lacked information about the neurochemical nature of the synapses that were either reduced or preserved.

A first indication that changes in the abundance of a specific set of synapses might contribute to the degeneration of motor neurons in ALS has come from immunohistochemical studies with the vesicular acetylcholine transporter, VAChT. VAChT immunoreactive synapses were largely depleted on surviving motor neurons in the spinal cords from patients with sporadic ALS, while synaptophysin-IR appeared unchanged on the same neurons

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(Nagao et al., 1998). It was concluded that loss of these cholinergic synapses might exacerbate excitotoxic motor neuron death.

The aim of the present study was to get a complete picture about the excitatory and inhibitory input on spinal cord motor neurons during disease progression in the SOD1 mouse model for ALS (Gurney et al., 1994) by assessing IR for different vesicular neurotransmitter transporter proteins as markers. The results obtained from this analysis should provide valuable information about the possibility that selective changes in synaptic input on motor neurons are a neuropathological hallmark in ALS that may contribute to motor neuron pathology.

## Methods

### *Animal model*

Male transgenic mice of the strain B6SJL-TgN(SOD1-G93A)1Gur (Jackson Laboratories, Bar Harbor, ME) that hemizygously carry the variant glycine to alanine at position 93 of the human Superoxide dismutase-1 gene (SOD1) in high copy number (Gurney et al., 1994) were used. They were housed individually under specific pathogen-free conditions in single ventilated cages and maintained at a constant temperature (22 ± 1°C) and a 12:12-h light/dark cycle with unrestricted access to food and water. All animal procedures were performed according to the German Animal Protection Law under an animal protocol approved by the county administrative government in Cologne. After a few days of habituation the SOD1 males were mated to background-matched control female mice of the strain B6SJLF1/J (Jackson Laboratories). Their progeny (first offspring) were genotyped by PCR for the presence of the transgene using gene-specific primers and a protocol recommended by the vendor (see http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protoco_id=523 for details).

Several SOD1 transgenic male progeny subsequently were bred to control females to obtain another generation of offspring for additional analyses (second offspring). All transgenic mice of ages 120 days and older showed a progressive paralysis of their hind limb muscles and died within 140–150 days of life, indicating that the transgene had been stably transmitted to the offspring. Female mice were used in all further experiments.

### *Motor performance*

Female mice from the first offspring were tested for their motor performance using a RotaRod device (Columbus Instruments, Columbus, OH). The mice were trained ten times daily on the first 3 days to become acquainted to the test. Thereafter, an observer blinded to the genotype tested them weekly. Each session consisted of three consecutive trials on the elevated RotaRod (8–25 rpm with an acceleration of 0.28 rpm/s starting after 20 s) and the longest latency to fall was recorded. 120 s was chosen as the arbitrary cutoff time. All data were normalized to the maximum value of the individual mouse because some control and some transgenic mice never achieved the cutoff time. The start of motor dysfunctions in individual SOD1 mice was recorded as the first day out of three consecutive test days where a mouse performed repeatedly worse than 90% of maximum.

The motor performance of female mice from the second offspring was analyzed with the paw grip endurance test (Weydt et al., 2003). This test is more sensitive to detect motor dysfunctions and immediately gives reliable results when compared to the RotaRod test. The mice were individually placed on a meshed platform that was attached to conventional bars at a height of 60 cm above the bench. The platform was gently flipped upside down and the latency determined until the mouse let loose with both hind limbs. Each mouse was given up to three consecutive attempts to reach the arbitrary maximum of 90 s and the longest latency was recorded.

All behavioral data were analyzed by factorial ANOVA, followed by Fisher's LSD post hoc test to detect differences between the groups.

### *Immunohistochemistry*

The mice were deeply anaesthetized by isoflurane inhalation and killed by cervical dislocation. The lumbar segments L3–L5 of the spinal cord were quickly dissected and immersion fixed for 3 days in Bouin Hollande fixative, containing 4% (w/v) picric acid, 2.5% cupric acetate, 3.7% formaldehyde, and 1% glacial acetic acid. Following fixation the tissues were extensively washed in 70% isopropanol, dehydrated, cleared with xylene, and embedded in paraffin. Seven-micron thick sections were cut with a microtome and mounted onto silanized glass slides.

The immunohistochemical staining of spinal cord sections was performed essentially as described previously (Schutz et al., 2000). Primary antibodies were used in these concentrations: rabbit anti-VAChT (1:15.000; Weihe et al., 1996); rabbit anti-vesicular monoamine transporter two, VMAT2 (1:8.000; Weihe and Eiden, 2000); rabbit anti-vesicular inhibitory amino acid transporter, VIAAT (1:2.000; Weihe and Eiden, 2000), guinea pig anti-vesicular glutamate transporter one, VGLUT1 (1:20.000; Varoqui et al., 2002), guinea pig anti-vesicular glutamate transporter two, VGLUT2 (1:10.000; Varoqui et al., 2002), and mouse anti-synaptophysin (1:20, clone SY38, Abcam, Oxford, UK). Immunoreactions were detected with species-specific biotinylated secondary antibodies (1:1.000; Dianova, Hamburg, Germany) and peroxidase reagents (Vectastain Elite ABC kit, Alexis, Grünberg, Germany) using 3,3′-diaminobenzidine and ammonium nickel sulfate as substrates. Finally, the sections were dehydrated and mounted under cover slips for microscopic analysis with an Olympus BX51 microscope. Digital images were processed with Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA).

The simultaneous detection of two vesicular transporters was achieved as described above with the following modifications: Primary antibody concentrations and combinations were goat anti-VAChT (1:1000) with rabbit anti-VIAAT (1:200), with guinea pig anti-VGLUT1 (1:1000), or with guinea pig anti-VGLUT2 (1:500), respectively. Antibody binding was visualized with secondary antibodies that were conjugated with fluorophores (MoBiTec, Göttingen, Germany). VAChT was visualized with donkey anti-goat-Cy3 conjugate (1:100), resulting in a red fluorescence. VIAAT, VGLUT1, and VGLUT2 were detected with species-specific antibodies labeled with Alexa488 conjugate (1:500), resulting in green fluorescent signals. The sections were mounted under cover slips with water based mounting medium (Biomeda, Foster City, CA) and kept at 4°C in the dark. Fluorescence was detected with a Leica DM IRBE inverted microscope (Leica, Bensheim, Germany), which was attached to a Leica TCS SP2 AOBS confocal system. Digital false color images for each